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(54) Title: DNA SEQUENCES ENCODING PROTEINS CONFERRING PHYTOPHTHORA INFESTANS RESISTANCE ON PLANTS

(57) Abstract: Genomic sequences encoding *Phytophthora infestans* resistance proteins are provided herein. Specifically, sequences from potato required for *P. infestans* resistance have been cloned and sequence provided, together with the encoded amino acid sequence. DNA encoding the amino acid sequence or amino acid sequences showing a significant degree of homology thereto may be introduced into plant cells and the encoded polypeptide expressed, conferring *P. infestans* resistance on plants comprising such cells and descendants thereof.

DNA sequences encoding proteins conferring *Phytophthora infestans* resistance on plants

FIELD OF THE INVENTION

This invention relates to methods and materials for improved plant disease resistance. In particular the present invention relates to nucleic acid sequences required for resistance of potato to *Phytophthora infestans*, recombinant polynucleotide molecules containing the sequences, and uses thereof to transform plants, especially plants of the family *Solanaceae* to make them more resistant to *Phytophthora* species.

BACKGROUND OF THE INVENTION

The oomycete pathogen *Phytophthora infestans*, is worldwide the main disease of the potato crop causing late blight that results in major losses of crop yield and quality. *P. infestans* infects plants of commercial importance like potato and tomato, that therefore require regular chemical control. Monogenic *R* genes have been introduced from the hexaploid Mexican wild species *Solanum demissum* into the cultivated tetraploid potato cultivars (Wastie, 1991). These race specific *R* genes did not provide durable field resistance because of the rapid evolution of new virulent races of the fungus that circumvent these *R* gene mediated resistances. Characteristic for *R* gene mediated resistance reactions is the hypersensitive response (HR) leading to local cell death causing necrotic spots at the site of attempted infection. Genetic analysis showed that activation of HR is highly specific and induced upon recognition by a specific *R* gene product and a corresponding avirulence gene product in the pathogen (Hammond-Kosack and Jones, 1997).

The *R* gene mediated resistance from wild *Solanum* species can show partial resistance or an intermediate HR response when crossed to different *S. tuberosum* backgrounds (Graham, 1963; Toxopeus, 1958). The HR lesions can vary in size depending on the backcross parent used, indicating that other genes influence the *R* gene resistance reaction. Minor *S. tuberosum* or *S. demissum* genes have been characterized to influence or even suppress *R* gene expression (El-Kharbotly *et al.*, 1996b). QTL mapping in *S. tuberosum* populations segregating for partial *P. infestans* resistance, identified 19 QTLs on 13 chromosomal regions (Leonards-Schippers *et al.*, 1994), with one QTL on chromosome 5 near the *P. infestans* resistance locus *R1* also linked to QTLs for maturity and vigor (Collins *et al.*, 1999). These QTLs on chromosome 5 very likely represent minor genes that play a role in both *R* gene mediated HR resistance responses

and developmental processes which indirectly influence the resistance response. Additionally, this chromosome region also contains several other resistance loci with specificity to different pathogens like the PVX virus (Ritter *et al.*, 1991) and potato cyst nematodes (Kreike *et al.*, 1994; Rouppe van der Voort *et al.*, 1998).

The cloning of *R* genes that mediate gene-for-gene type resistance to bacterial, fungal, oomycete, viral, and nematode pathogens has so far identified 5 classes of genes based on common characteristics including nucleotide binding sites, leucine-rich repeats, transmembrane domains and serine/threonine protein kinases (Hammond-Kosack and Jones, 1997). Genetic mapping and sequence analysis showed frequent clustering of *R* genes with different resistance specificities at complex loci (Jia *et al.*, 1997; Parniske *et al.*, 1997). Despite these insights into *R* gene structure their function can not be predicted from sequence alone and functional tests are required to determine their role in resistance (Parker *et al.*, 1996).

A few *R* gene signal transduction components have been identified by mutation (reviewed in Innes, 1998). These analyses have helped identify genes that are required for the barley powdery mildew mediated *Mla-12* resistance (*rar-1* and *rar-2*; Jørgensen, 1996), the tomato *Pseudomonas syringae* pv *tomato* resistance gene *Pto* (*Prf*; Salmeron *et al.*, 1996) and *Pti*; (Zhou *et al.*, 1995) and for the tomato *Cf-9* (*rcr-1* and *rcr-2;* Hammond-Kosack and Jones, 1994) and *Cf-2* mediated *Cladosporium fulvum* resistance reactions (*rcr-3;* Jones *et al.*, 1999). Extensive mutant screens in Arabidopsis identified a number of genes involved in plant pathogen interactions, *ndr1* (Century *et al.*, 1995), *eds1* (Parker *et al.*, 1996), *pad1*, *pad2*, *pad3* and *pad4* (Glazebrook *et al.*, 1996) and *pbs1*, *pbs2* and *pbs3* (Warren *et al.*, 1999). Most of these mutations affect the function of a subset of *R* genes (Aarts *et al.*, 1998) or only combinations of double mutations significantly decrease *R* gene resistance (Glazebrook *et al.*, 1997; Warren *et al.*, 1999; McDowell *et al.*, 2000). This indicates the occurrence of different signaling pathways for resistance reactions that are also partially redundant.

Transposon tagging is an established tool in plants for the identification of genes that display a mutant phenotype when their function is disrupted. Transposons have been introduced from maize and successfully used for tagging in many heterologous plants like Arabidopsis (Aarts *et al.*, 1993), petunia (Chuck *et al.*, 1993), tobacco (Whitham *et al.*, 1994), tomato (Jones *et al.*, 1994) and flax (Lawrence *et al.*, 1995). In these self-fertilizing plant species random tagging strategies (Arabidopsis, petunia) by screening large selfed populations for mutants or targeted tagging of specific genes (tobacco, tomato, flax) were applied. By self- or test- crossing, large populations were produced for the direct screening of possible transposon tagged mutants. By using selectable markers like kanamycin (Baker *et al.*, 1987) or hygromycin (Rommens *et al.*,

1992), selection of excision events at the cellular level has been feasible and in combination with effective in vitro selection and somatic propagation procedures can facilitate the production of large numbers of transposon insertion mutants.

SUMMARY OF THE INVENTION

The present invention provides an isolated DNA sequence which encodes a protein having the amino acid sequence given in SEQ ID NO:2 or a functionally homologous protein having an amino acid sequence showing an identity of at least 80% to SEQ ID NO:2, which protein confers *Phytophthora infestans* resistance on plants.

Preferably, the DNA sequence encodes a protein having an amino acid sequence showing an identity of at least 85%, or even 90%, to SEQ ID NO:2.

More preferably, the DNA sequence encodes the amino acid sequence given in SEQ ID NO:2, in which case the DNA sequence comprises the nucleotide sequence given in SEQ ID NO:1.

More general, the invention provides the DNA sequence selected from the group consisting of:

a) the DNA sequence given in SEQ ID NO:1 and its complementary strand, and

b) DNA sequences hybridizing to the sequences in (a) under stringent hybridization conditions.

In particular the DNA sequence comprises the nucleotide sequence of nucleotides 20 to 1053 of SEQ ID NO:1, which is the coding sequence for the protein having the amino acid sequence given in SEQ ID NO:2. Said nucleotide sequence contains an intron, nucleotides 584 to 726. Accordingly, the actual DNA sequence encoding the protein of SEQ ID NO:2 comprises nucleotides 20 to 583 and 727 to 1053 of SEQ ID NO:1.

In a further aspect the invention provides a protein having the amino acid sequence given in SEQ ID NO:2 or a functionally homologous protein having an amino acid sequence showing an identity of at least 80% to SEQ ID NO:2, which protein confers *Phytophthora infestans* resistance on plants.

In another aspect the invention provides a recombinant vector comprising a DNA sequence as defined above under control of an appropriate promoter and regulatory elements for expression in a host cell.

In still another aspect the invention discloses the use of the present DNA sequence or recombinant vector for the production of a transgenic plant.

Further the invention provides a host cell, preferably a plant cell, comprising the present DNA sequence or recombinant vector.

Also provided is a plant or any part thereof comprising such plant cell, and seed, selfed or hybrid progeny or descendant of such a plant, or any part thereof.

The invention also provides a method of conferring *Phytophthora infestans* resistance on a plant, comprising the steps of

i) introducing a DNA sequence as defined above or a recombinant vector as defined above into a cell of the plant or an ancestor thereof,

ii) regenerating plants from the obtained transgenic cells, and

iii) selecting plants exhibiting *P. infestans* resistance.

A further aspect of the invention is the provision of oligonucleotide probes that comprise a sequence of nucleotides of SEQ ID 1, or a mutant, derivative or allele thereof, capable of detecting the pathogen resistance gene or functional equivalents thereof in plants of the family *Solanaceae* and the use of the probes to isolate DNA sequences encoding a pathogen resistance gene or a functional equivalent thereof.

Using the sequence SEQ ID 1 facilitates the isolation of homologous genes from related and unrelated hosts to obtain genes, which protect host plants against related and unrelated pathogens.

A further aspect of the invention is the identification of proteins that interact with constructs comprising sufficient homology to SEQ ID 2, the genes thereof can be used to provide plant cells that are resistant to pathogens. One way is by identification of interacting proteins by the yeast two-hybrid system that are then involved in the signal transduction of the resistance response.

A further aspect of the invention is the construction of hybrid proteins comprising SEQ ID 2 or DNA isolates of sufficient homology, with other proteins that can be used as effector molecules. One way is by making hybrids with different leucine rich repeat fragments from various plants or synthetically produced *in vitro*, that can interact with different pathogen or inducer effector molecules. These effectors can also be chemically produced and by application to a plant containing the hybrid construct can induce the signal transduction pathway for resistance.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Schematic drawing of pHPT::*Ds*-Kan showing positions of primer 1 (p1, GCG CGT TCA AAA GTC GCC TA), primer 2 (p2, GTC AAG CAC TTC CGG AAT CG) and *PstI* restriction sites. Abbreviations: LB = left border, RB = right border, pNOS = nopaline synthase promoter, NPT II = neomycin phosphotransferase gene, HPT II = hygromycin phosphotransferase gene.

Figure 2: *PstI* restriction of genomic DNA hybridized to NOS promoter probe to select for presence of full donor site (FDS = 4.0 kb), empty donor site (EDS = 2.3 kb), *Ac* T-DNA construct (3.5 kb) and *Ds* re-insertion sites in the R1Ds/r-; Ac/- selected seedlings EE96-4311-37 (lane 1), EE96-4312-43 (lane 5) EE96-4312-49 (lane 9) and HygR protoplast regenerants from EE96-4311-37 (lane 2,3 and 4), EE96-4312-43 (lane 6, 7 and 8), EE96-4312-49 (lane 10, 11, 12 and 13), EE96-4311-15 (lane 13, 15), EE96-4312-05 (lane 14), EE96-4312-76 (lane 16) and EE96-4312-06 (lane 17).

Figure 3: Reaction phenotypes observed on different genotypes after inoculation of detached leaves with *P. infestans* race 0. a) TM17-2, susceptible parent; b) detail of sporulation on TM17-2; c) HRPR 836; d) HRPR 1587 showing both the *R1* type HR response and necrotic regions with sporulation; e) detail of HR spot on HRPR 1587; f) detail of sporulation on the necrotic region of HRPR 1587; g) necrotic regions on R1Ds/r-; Ac/- seedling EE96-4312-03, minor sporulation was detected in such regions; h) clear colonization on variant 1000; i) detail of sporulation on variant 1000.

Figure 4: *Hin*dIII digested genomic DNA hybridized to the 5' Ac probe (A) or the internal *Ac* probe (B). Lane 1 shows the 1.6-kb marker hybridization. The *R1* resistant crossing parent J91-6167-2 (lane 2a and b) and the susceptible crossing parent 87-1024-2 (lane 3a and b) contain both no *Ac* or *Ds* elements. The primary transformant Ds416 contained two *Ds* T-DNA loci (lane 4a), Ds53-34 inherited both *Ds* T-DNA loci (lane 5a) as did EE96-4312-28 (lane 7a). EE96-4312-28 inherited from TM17-2 (lane 6a) the *Ac* element. In mutant 487 (lane 8a) and mutant 1000 (lane 9b), both regenerated from EE96-4312-28, the *Ds* elements transposed to new positions and *Ac* seems to be missing. In TM17-2 (lane 6b) a complete *Ac* (1.6 kb internal *Hin*dIII fragment) and a d*Ac* (2.9 kb) are present. Mutant 487

(lane 8b) inherited d*Ac* as a different restriction fragment due to the insertion of *Ac* in d*Ac*. In mutant 1000 (lane 9b) *Ac* got lost and only d*Ac* is present.

Figure 5: a) Schematic representation of the isolated *Ds* flanking sequences from mutant 1000 (*rpr1* and *rpr2*) and their alignment to XA21 aa 708- 1011. The large triangles represent the *Ds* positions, the intron region is a dashed line and the small black triangles represent primer positions of EE1 (5'- ACA TTG GGC ACT CTT GGA TAC A), EE2 (5'- TCT TGA TTC TGG CAT TTT CTT TG), EE3 (5'- CCT GAC ACA AAC CGA GAC ATT), EE6 (5'- AAC AAT GCC TTT CTT CTC), EE8 (5'- GCA CAT TAT CAA GTG GAA CTA CG) and EE10 (5'- CTG AGC CGT ACT CTT AAA AGA ACG). b) Amino acid alignment of Pto, Pti1, StPK-B, StPK-A and Xa21. The eleven conserved domains of a protein kinases are numbered and the conserved amino acids are marked (*). Bold domains are specific for serine/threonine recognition. The N-glycosylation site is underlined.

Figure 6 (A and B): Sequence of the StPK-B DNA (SEQ ID NO: 1) and StPK-B protein (SEQ ID NO: 2). StPK-B DNA sequence is the contiguous sequence of a DNA fragment obtained by isolating the Ds tagged gene. The sequence is part of the StPK-B gene encoding the protein kinase domain and lacks the N-terminal portion of the gene including the translation start. SEQ ID 2 is the amino acid translation of SEQ ID 1 after removing the predicted intron conserved with other gene family members.

Figure 7: DNA sequences of StPK homologs obtained by using primers EE1 and EE2. SEQ ID 3 is the sequence of DNA fragments of StPK-A that is tagged by the transposon Ds. SEQ ID 4-12 are DNA fragments obtained by PCR between primers EE1 and EE2; Seq ID 4-12 represent fragments of homologous sequences StPK-C to StPK-K, in the genome.

Figure 8: Diagram of StPK overexpression constructs used for plant transformation. The StPK-B gene with a synthetic translation initiation start, under control of the CaMV 35S promoter and Nos-terminator is cloned in a binary vector for transformation of plants using Agrobacterium tumefaciens strains in construct I. Construct II contains the N-terminal part of the StPK genes or other N-terminal fusions to protein domains that act as recognition domains with other effector molecules.

DETAILED DESCRIPTION OF THE INVENTION AND EXPERIMENTS

Development of transposon mutagenized potato plants

Diploid potato plants heterozygous for the *P. infestans R1* resistance gene were transformed with an Agrobacterium strain containing a *Ds*-transposon T-DNA construct shown in Figure 1 (Pereira *et al.*, 1992; El -Kharbotly *et al.*, 1995). Transformant Ds416 contained a *Ds* T-DNA insertion on chromosome 5 (El-Kharbotly *et al.*, 1996a), linked in repulsion phase to the previously mapped *P. infestans R1* resistance gene (Leonards-Schippers *et al.*, 1992). This Ds416 clone was crossed to the susceptible diploid genotypes J89-5040-2 producing offspring that enabled the selection of recombinant plants (Ds53-22 and -34) having the *R1* gene and the *Ds* T-DNA in coupling phase (18 cM) (El-Kharbotly *et al.*, 1996a). To activate the *Ds* transposon these plants were crossed with TM17-2, a diploid potato clone susceptible to *P. infestans* and transformed with the *Ac* transposon-containing T-DNA construct pMK1GBSS*Ac* (Pereira *et al.*, 1991). TM17-2 contained one functional *Ac* displaying active transposition. From the progeny of these crosses, population EE96-4311 (Ds53-22 X TM17-2; 18 seedlings) and EE96-4312 (Ds53-34 X TM17-2; 96 seedlings), 47 (8 and 39) kanamycin resistant *R1* seedlings (KanR *R1*) were selected.

Plant genomic DNA was isolated from greenhouse grown leaves (Pereira and Aarts, 1998) and used for molecular analysis. Empty donor sites (EDS-PCR) indicating excision were detected in 22 of the 47 KanR *R1* seedlings as a 450-bp PCR product using specific primers (Figure 1) and confirmed by Southern blot analysis. After selection of the 22 *R1* resistant seedlings showing active *Ds* excision (R1Ds/r-; Ac/-), the expression of hygromycin resistance (HygR) was tested by rooting on MS30 supplemented with 10-100-mg/l hygromycin. One genotype EE96-4311-12 showed resistance by rooting on 40 mg/l hygromycin and displayed a clear EDS fragment, suggesting that screening for rooting of shoots on 40 mg/l could be used as a stringent criteria for *Ds* excision.

As most genotypes contained excision events that occurred late in shoot development these HygR cells could be selected by protoplast isolation and screening for hygromycin resistance to select independent excision events. Protoplasts were isolated from 4-week-old *in vitro* grown shoots (Uijtewaal *et al.*, 1987), re-suspended in culture medium TM2G (Wolters *et al.*, 1991) to a final concentration of 500,000 pp/ml and diluted weekly with fresh medium. The regenerating calli were progressively transferred to callus growth medium, shoot induction medium and finally maintained on shoot elongation medium until regenerated plants could be harvested (Mattheij *et al.*, 1992). In separate experiments to

select specifically for protoplast regenerants with excision events, 10 mg/l hygromycin was added to the callus growth medium 14 days after protoplast isolation, then increased to 20 mg/l on day 21 and maintained at this level.

Table 1 gives an overview of protoplast regeneration data. From parental clone Ds53-34, control EE96-4312-21 and selected R1Ds/r-; Ac/- seedlings about 50 regenerating shoots were tested for their rooting ability on MS30 with 40 mg/l hygromycin. As expected, the parent Ds53-34 and control EE96-4312-21 produced no HygR protoplast regenerants whereas EE96-4311-12 gave 45% HygR protoplast regenerants confirming early excision. The other 14 good performing R1Ds/r-; Ac/- plants showed regeneration of 4 to 33% of HygR shoots indicating excision of *Ds* from its original T-DNA location. The use of hygromycin selection during callus culture and regeneration of shoots increased the recovery of HygR regenerants 3.8 times. A total of 1973 HygR regenerants were obtained from different selection experiments and transferred to the greenhouse.

Table 1: Selection of excision events after protoplast regeneration with and without hygromycin selection. Number of calli, shoots and selected hygromycin resistant (HygR) regenerants for parents Ds53-22 and Ds53-34; control EE96-4312-21 (R1Ds/r-; -/-) and 22-selected R1Ds/r-; Ac/- genotypes from the seedling populations EE96-4311 and EE96-4312.
[a] low due to infection.

Genotype	No selection During protoplast regeneration			Hygromycin selection During protoplast regeneration		
	Calli	Shoots	HygR	Calli	Shoots	HygR
Ds53-22	10	0		47	0	
Ds53-34	100	45	0	134	1	0
EE96-4312-21	100	21	0	900	10	0
EE96-4311-08	0[a]			0[a]		
EE96-4311-12	100	49	22	1000	198	98
EE96-4311-15	300	82	11	800	160	101
EE96-4312-03	100	23	3	1000	166	83
EE96-4312-05	100	29	8	1000	198	121
EE96-4312-06	100	6[a]	2	1000	205	139
EE96-4312-14	100	70	15	1000	208	118
EE96-4312-23	100	51	2	1000	211	91
EE96-4312-27	10	0		10	0	
EE96-4312-28	100	47	7	1000	143	82
EE96-4312-30	100	0		419	0	
EE96-4312-31	100	30	2	570	21	4
EE96-4312-37	100	52	2	1000	248	92
EE96-4312-40	33	2	0	67	0	
EE96-4312-43	100	45	8	650	207	101
EE96-4312-46	14	9	0	103	0	
EE96-4312-49	100	48	3	1000	206	109
EE96-4312-52	3	3	0	1	1	1
EE96-4312-60	100	52	7	1000	203	93
EE96-4312-63	100	50	7	1000	203	130
EE96-4312-76	0	0		24	0	
EE96-4312-89	100	49	4	274	41	19
Total		691	103		2619	1382

To analyze *Ds* excision in the HygR protoplast regenerants Southern blot hybridization was performed on a subset of selected R1Ds/r-; Ac/- seedlings and some of their HygR protoplast regenerants (Figure 2). Plant DNA was restricted with *Pst*1 and the blots hybridized to probes derived from the NOS promoter fragment that revealed the *Ac* T-DNA and the *Ds* transposon. The R1Ds/r-; Ac/- seedlings used for protoplast isolation all displayed two *PstI* fragments, respectively 4.0- and 3.5-kb, corresponding to respectively the *Ds* T-DNA and the *Ac* T-DNA constructs. Faintly visible fragments of 2.3-kb were also detected that correspond to a low amount of EDS fragments present in these seedlings. All

HygR protoplast regenerants showed a strong hybridizing EDS fragment indicating early or repeated excision of *Ds* corresponding to the high level of hygromycin resistance for which these plants were selected. The original *Ds* parent had two copies of *Ds* at one locus. Full donor site fragments were detected in most of the HygR protoplast regenerants which indicates that one of the two *Ds*'s was not excised. Three plants shown in Fig 2, showed a complete EDS indicating that excision occurred in the initial protoplast. Most HygR regenerants showed clear *Ds* re-insertion fragments varying from 1 to 8 new positions per individual HygR regenerant. Regenerants from a single seedling showed different re-insertion patterns, indicating that they originated from independent transposition events and confirmed that most selected HygR regenerants originate from independent transposition events.

The somatic selection of *Ds* transpositions from individual cells facilitated the production of a large population of shoots with independent *Ds* excision events. The HygR protoplast regenerants potentially represent about 2000 independent *Ds* insertions. This number of *Ds* insertion mutations should be enough for the isolation of tagged mutants involved in *R1* resistance. The somatic selection of *Ds* transposition and the rapid production of independent plants containing these transpositions, facilitates the production of large tagging populations needed for the transposon mutagenesis of selected genes. This is particularly suitable for the mutagenesis of genes in heterozygous crops like potato.

Screen for *R1* type HR resistance variants in the *Ds* tagged population

The transposon mutagenized population was suitable for the isolation of mutations in defense related genes causing an altered reaction to *P. infestans*. By using a suitable screen quantitative changes towards susceptibility were possible to be identified. Race specific resistance *Cf* genes in tomato have shown a semidominant phenotype if screened in a quantitative manner (Hammond-Kosack and Jones, 1994). Chromosome 5 in potato is known to contain many resistance components (Leonards-Schippers *et al.*, 1994) that are probably in a heterozygous state as seen from segregation of minor factors. These loci could probably be efficiently mutagenized due to active linked transposition of *Ds* near *R1*.

To prepare the inoculum for screening (El-Kharbotly *et al.*, 1994), *P. infestans* race 0 (89148-09) was grown on rye agar medium (with 20 mg/l sucrose). The sporangiospores were washed with 10-15 ml cold tap water (4°C) and the resulting suspension used to inoculate 10 Bintje tuber slices (1 cm thickness). The newly formed sporangiospores were washed and again used to inoculate 20-50 tuber slices of Bintje in order to obtain 1-2 l of

sporangiospore solution. This solution was diluted to contain at least 2000 spores/ml to use for plant inoculation.

The 1973 hygromycin resistant protoplast regenerant (HRPR's) plants to be tested were periodically brought in batches to the greenhouse. After 6-10 weeks growth two leaves of each HRPR plant were harvested, placed in columns of water absorbent substrate, and put in containers (46 x 31 x 8 cm) closed with transparent covers. In every container two leaves of 10 HRPR plants and a leaf of the susceptible control (Bintje or TM17-2) were tested. In each experiment 15-30 containers were used so that 150-300 plants could be tested in parallel, with Ds53-22, Ds53-34 and TM17-2 always tested as additional controls. Each leaf in the experiments was sprayed with about 5-10 ml of the sporangiospore solution containing 10,000-50,000 sporangiospores. After 5 days in high humidity at 16°C, all leaves were evaluated for the development of *P. infestans* infection symptoms and at day 6 a second evaluation for disease symptoms was performed. When development of symptoms occurred the leaves were kept for an additional 2 days for a microscopic examination of the disease development.

The susceptible parent and control cultivar Bintje always showed distinctive colonization and abundant sporulation on day 5-6 (Figure 3a and 3b). In contrast the resistant parents Ds53-22, Ds53-34 and most of the analyzed HRPR's always displayed characteristic *R1* type HR spots upon infection. The phenotype of HRPR 836 was distinctly susceptible with colonization and sporulation over large leaf areas (Figure 3c). Other HRPR's sometimes showed larger necrotic regions indicating colonization of the leaves (Figure 3d). When this colonization resulted in sporulation (Figure 3f) the HRPR was scored as a potentially susceptible *R1* variant, although necrotic spots were additionally visible on the green parts (Figure 3e) indicating at least a partial HR activation. In this first round of screening 33 putative susceptible variants, derived from 10 R1Ds/r-; Ac/- seedlings were selected (Table 2). Re-inoculation tests of newly grown leaves of the selected variants confirmed the susceptible reactions for 9 variants.

Table 2: Primary screen for mutants with an altered *R1* type HR resistance response.

R1Ds/r-;Ac/- Seedling	total # selected HRPR's	# HRPR's tested with *P. infestans* race 0	# HRPR's Variant	Variant plant #	Ploidy level
EE96-4311-12	126	72	1	702	4x
EE96-4311-15	112	81	2	35	2x
				994	2x
EE96-4312-03	86	63	2	1515*	2x
				1921	nd
EE96-4312-05	129	84	2	836*	4x
				842	4x
EE96-4312-06	243	188			
EE96-4312-14	195	168	2	925	4x
				1587	2x
EE96-4312-23	93	82			
EE96-4312-27	5	0			
EE96-4312-28	89	71	7	487*	2x
				998	2x
				999	4x
				1000	2x
				1001	4x
				1005	4x
				1357	2x
EE96-4312-31	7	4			
EE96-4312-37	134	120	4	151*	4x
				510*	4x
				524	2x
				551	4x
EE96-4312-43	109	91	6	570*	4x
				688*	4x
				1528	4x
				561	x-4x
				562	x-4x
				574	4x
EE96-4312-49	152	111	6	600	4x
				601	4x
				633*	2x
				1050	2x
				1055*	4x
				1073	4x
EE96-4312-52	1	1			
EE96-4312-60	134	112	1	667	4x
EE96-4312-63	168	155			
EE96-4312-76	83	65			
EE96-4312-89	107	96			
Total	1973	1564	33		

= number, nd = not determined, * variants with confirmed susceptible reaction after re-inoculation.

Selected genotypes were transferred from the greenhouse to *in vitro* for propagation to obtain 10 or 35 cuttings of each variant and these were transferred again to the greenhouse for a replicated re-testing of the *P. infestans R1* resistance. From the first set of 33 *R1* variants, ploidy level analysis enabled the identification of plants with chromosomal anomalies that were potentially somaclonal variants. All the diploid variants together with those with a reproducible susceptible phenotype and the corresponding 9 parental seedlings were used for a secondary quantitative phenotypic analysis. After *P. infestans* inoculation on two leaves of each plant, the developing symptoms were carefully evaluated and followed microscopically when necessary to detect sporulation (Table 3).

Table 3: Qualitative and quantitative analysis of the resistance reaction and disease development on selected variants compared to parental controls.

	# plants	% leaves with HR	% leaves with <20% necrosis and sporulation	% leaves with 20-100% necrosis, colonization sporulation (max % leaf area covered)		& Dead (Rotten)
Parents						
Ds53-34	10	100	0	0		0
TM17-2	10	0	0	100	(100)	0
R1Ds/r-; Ac/- Seedlings						
EE96-4312-76	10	100	0	0		0
EE96-4312-43	9	94	6	0		0
EE96-4312-37	19	87	13	0		0
EE96-4312-28	20	75	23	2	(35)	0
EE96-4312-49	10	75	20	5	(25)	0
EE96-4312-05	20	75	15	5	(40)	5
EE96-4312-03	21	71	19	10	(60)	0
EE96-4311-15	20	60	35	5	(50)	0
EE96-4312-14	19	47	18	32	(100)	3
Variants						
EE96-4312-43 570	9	100	0	0		0
688	7	100	0	0		0
EE96-4312-37 510	10	95	5	0		0
524	10	90	5	0		5
EE96-4312-28 487	35	41	43	16	(100)	0
998	34	60	38	2	(35)	0
1000	35	16	34	50	(100)	0
1357	34	63	24	9	(70)	4
EE96-4312-49 601	10	60	30	10	(60)	0
633	10	70	25	5	(60)	0
1050	9	78	17	5	(45)	0
1055	10	80	10	5	(50)	5
EE96-4312-05 836	34	7	12	68	(100)	13
EE96-4312-03 1515	31	76	16	8	(50)	0
EE96-4311-15 35	14	82	0	7	(50)	11
994	8	44	0	56	(100)	0
EE96-4312-14 1587	1	50	0	50	(60)	0

= number, HR = hypersensitive response

The *R1* resistant parental plant Ds53-34 always showed the complete *R1* type HR response, with small necrotic spots on inoculated leaves. The *R1* resistant progeny of Ds53-22 and Ds53-34 (EE96-4311-15 and EE96-4312-03, 05, 14, 28, 37, 43, 49 and 76) displayed an intermediate resistance phenotype (Table 3). With the exception of seedling EE96-4312-76, all other seedlings showed on several leaves (6-35%) necrotic spots that developed into necrotic regions covering 5 to 20% of the leaf area (Figure 3g). Microscopic examination revealed very little sporulation in these regions indicating minor escape of *P. infestans* from the normal *R1* type HR response. In a few leaves the necrotic region covered almost 100% of the leaf area and colonization with sporulation was observed indicating susceptibility of the leaf and escape from the *R1* type HR resistance response. Seedling EE96-4312-14 showed in this analysis only in 47% of the leaves a clear *R1* type HR response. However, from the 168 HRPR's derived from this seedling and tested in the first screening for *R1* resistances only 2 were selected as putative variants (Table 2). This indicates that the intermediate phenotype for this and other parental seedlings did not result in an overestimation of putative variants in the first screening.

The re-evaluation of the resistance response reaction for the variants 487, 1000, 836 and 994 showed a clear deviation in phenotype when compared to the parental seedlings. Variant 1000 showed colonization and sporulation on 50% of the leaves and this clearly resembled the TM17-2 *P. infestans* susceptible parental phenotype (Figure 3h and 3i). Only 16% of the variant 1000 leaves showed the normal *R1* type HR resistance response. In variant 487 the *R1* type HR resistance response was clearly detected in only 41% of the inoculated leaves. On 16% of the leaves necrotic regions covered over 20% of the leaf area and colonization and sporulation was detected indicating a weak susceptible *R1* variant.

The susceptible phenotype of variant 836 in the first screening of the HRPR population (Figure 3c) was repeatable in this analyses but quick senescence of the leaves, resulting in softening and rotting, suggested other causes for the observed susceptible phenotype. Variant 994 showed a striking phenotype as in every plant the youngest leaf showed colonization with sporulation, combined with leaf softening and rotting. The second oldest leaf analyzed always showed a normal *R1* type HR resistance phenotype. The variants 836 and 994 therefore displayed a more susceptible reaction due to interaction with early senescence and were therefore not considered as variants in the expression of *R1* type resistance. Re-evaluation of the resistance phenotype of the variants 570, 688, 510, 524, 633, 1050, 1055, 1515 and 35 did not reveal any quantitative difference when compared to the parental seedlings and were not regarded as mutants in the *R1* resistance reaction.

Molecular analysis of the tagged mutants

To examine the causal relationship between the *Ds* insertion sites and the observed phenotype mutant 1000 was characterized by Southern blot hybridization. Genomic DNA from appropriate genotypes was restricted with *Hin*dIII and the blots hybridized to a 5' *Ac* probe to determine the presence and positions of the *Ac* and *Ds* elements in the different genotypes. Since *Ds* is derived from *Ac*, the 5'*Ac* probe also identified the *Ds* element (Pereira *et al.*, 1992). Additional hybridization of the same blots with an internal *Ac* probe revealed the presence and position of *Ac*. In the different genotypes the *Ds* and *Ac* insertions were identified by specific *Hin*dIII fragments (Figure 4a and 4b). New positions of the *Ds* elements confirmed that both mutants (from same seedling parent) were derived from different *Ds* transposition events in EE96-4312-28 during protoplast regeneration (Fig 4a). These hybridizations also revealed that mutant 1000 had lost the *Ac* element and was therefore a stable mutant.

To analyze the sites of *Ds* insertion, flanking DNA of *Ds* insertions was isolated by inverse PCR (IPCR ;Triglia *et al*, 1998). Plant genomic DNA, was restricted with *Hae*III, self-ligated and restricted with *Bam*HI and *Bgl*II. *Bgl*II restriction prevents the amplification of the *Ds* transposon flanking sequences in the original T-DNA construct. To obtain additional and longer 5' flanking sequences a second restriction combination was used, in which genomic DNA was restricted with *Msc*I followed by *Hin*dIII and after ligation linearized by *Cla*I. Primer 5'-CGG GAT GAT CCC GTT TCG TT (*Ac* position 197-216) and primer 5'-GAT AAC GGT CGG TAC GGG AT (*Ac* position 44 - 35) were used to amplify the 5' *Ds/Ac* flanking sequences. After a hot start (10 min 94°C), 35 PCR cycles (1 min 94°C, 1 min 60°C, 2 min 72°C) resulted in the amplification of *Ac* and *Ds* 5' flanking sequences.

Thermal asymmetric interlaced (TAIL) PCR (Liu and Whittier, 1995) was used to obtain additional *Ds* transposon flanking sequences. Sets of nested primers designed on the 5'- and the 3' site of the *Ac* transposon (Tsugeki *et al.*, 1996; Ds5-1, 5-2, 5-3, 5-4 and Ds3-1, 3-2, 3-3, 3-4) were combined with 4 different degenerated primers (AD1 to 4; Liu and Whittier, 1995) or two other degenerate primers (Tsugeki *et al.*, 1996; renamed AD5 and 6). The three step PCR reactions were performed as described (Tsugeki *et al.*, 1996). Primers AD3, AD5 and AD6 with *Ac/Ds* 5' primers and primers AD5 and AD6 with *Ac/Ds* 3' primers produced specific PCR fragments.

The IPCR and TAIL-PCR products were separated on a 1% TBE agarose gel to determine the number and size of fragments. After phenol:chloroform extraction and isopropanol precipitation, the PCR products were cloned in pGEM -T easy vector (Promega Corporation). For each sample three clones were sequenced using an automated ABI 373 DNA sequencer. The obtained *Ds* flanking sequences were compared to known sequences by BlastN and BlastX homology searches (Altschul *et al.*, 1997) in the public databases.

In mutant 1000 two different *Ds* insertions were identified by BlastX searches, each with homology to a leucine rich repeat containing protein kinase from *Oryza longistaminata* (Tarchini *et al.*, 2000) and a receptor protein kinase-like protein from *Arabidopsis thaliana* (BAC clone F13I12). These sequences were both identified due to their homology to the serine/threonine kinase domain of the *Xanthomonas* resistance gene *Xa21* isolated from *O. longistaminata* (Song *et al.*, 1995). Additional *Ds* flanking sequences were isolated using TAIL-PCR (540 bp; Fig 5a). Combining the 5' 288 bp and the 3' 540 bp flanking sequence for this *Ds* insertion revealed the expected 8-bp target site duplication. The sequence flanking this *Ds* insertion showed 50% protein identity to XA21. For the second *Ds* insertions the IPCR and TAIL-PCR only extended the 5' flanking sequence from 496 to 1309 bp (Fig 5a). This sequence covered a complete serine/threonine protein kinase ORF (nucleotides 20 to 583 and 727 to 1053) with 44% identity to the serine/threonine protein kinase domain of XA21 including the conserved intron position , nucleotides 584 to 726 (Fig 5a). All eleven protein kinase specific domains with conserved features were present (Fig 5b) as well as all the 14 conserved amino acids (Hanks *et al.*, 1988). Domain VI (consensus DLKPEN) and domain VIII (consensus G(T/S)XX(Y/F)XAPE) are indicative of serine/threonine specificity (Hanks *et al.*, 1988). The two *Ds* insertion loci displayed 84% identity at the protein level while in the intron region they showed only 52% nucleotide identity. This was a clear indication that the isolated *Ds* flanking *Solanum tuberosum* protein kinase (StPK) represented two distinct *Ds* tagged loci in mutant 1000, StPK-A and StPK-B. Fig. 6(A) shows the nucleotide sequence of StPK-B (SEQ ID NO: 1) and Fig. 6(B) shows the deduced amino acid sequence of StPK-B (SEQ ID NO: 2).

Although the protein identity was less than 50%, all characteristic protein kinase domains and conserved amino acids were present in the potato insertion loci StPK-A and StPK-B (except for domain X and XI in StPK-A), including the intron position at exactly the same position in the serine/threonine specific domain VIII. Therefore, it is probable that these serine/threonine protein kinases are similarly functional in the signal transduction pathway leading to *P. infestans* resistance and perhaps other pathogens.

Surprisingly, the StPKs are more homologous to the protein kinase domain of the rice resistance gene *Xa21* than to the earlier identified *Solanaceous* tomato resistance genes *Pto* (Martin *et al.*, 1993) and *Pti* (Zhou *et al.*, 1995). More surprisingly, since a homologue of *Pto* was mapped to the *R1* chromosomal 5 area (Leister *et al.*, 1996). These tomato serine/threonine kinases are functional in the signal transduction pathway leading to a hypersensitive response reaction upon infection with *Pseudomonas syringae* pv. tomato strains expressing the avirulence gene *avrPto* (Zhou *et al.*, 1997). In *Xa21*, other rice homologs (Tarchini *et al.*, 2000) and in the StPK-A and StPK-B the conserved intron position in domain VIII indicates a conserved gene family among monocots and dicots. No intron position was identified in the tomato serine/threonine kinases *Pto* and *Ptil*. Among the 11 kinase specific domains only minor differences were observed between the potato kinases and *Xa21* on one hand and *Pto* and *Ptil* on the other hand (Fig 5b). But overall amino acid homology determined that the potato sequences were more related to *Xa21* than to the tomato kinases *Pto* and *Ptil*. Domain IV, with no general consensus, showed a high homology between *Xa21* and the potato sequences while *Pto* and *Ptil* contained different amino acids in this area. Whether this difference determines a clear difference in function or signaling pathway for these kinases needs to be studied.

Identification of potato protein kinase (StPK) homologs

To characterize the structure of the StPK homologs in *R1* resistant and susceptible plants several sets of primers were designed and used in PCR analysis. Primers EE1 and EE2 (Fig 5a) could amplify a product of expected size of about 470 bp and a second product of about 370 bp from the *R1* resistant parent J91-6167-2, the susceptible parent 87-1024-2 and several *R1* resistant and susceptible progeny (J92-6400-A1, -A2, -A3, -A4, -A5 and -A6). Sequencing the PCR products derived from J91-6167-2, 87-1024-2, J92-6400-A1 and -A4 identified 10 different StPKs (Table 4). In Fig. 7 the DNA sequences of the StPK homologs are shown (partial sequences of the PCR products), SEQ ID NO:3-12. StPK-A was not identified in any of the plants by using this primer combination. From the susceptible parental clone 87-1024-2, StPK-B was isolated. Two additional StPK homologs, StPK-C and -D were identified several times in both *R1* resistant and susceptible clones. StPK-D was the 370 bp PCR product and had a deletion of 108 bp making it very likely a non-functional StPK. From the *R1* resistant plants two additional StPK homologs, StPK-E and -I were isolated and from the susceptible plants 5 additional homologs were isolated, StPK-F, -G, -

H, -J and -K (Table 4). The sequences of StPK-F and StPK-G shown in Fig. 7 are smaller than they actually are due to sequencing problems. The isolation of these many StPK homologs indicated that these serine/threonine protein kinases represent a multigene family in *S. tuberosum*. This was confirmed by DNA hybridization, since StPK-B identified over 30 hybridizing fragments in *Hin*dIII or *Eco*RI digested genomic DNA of a single resistant or susceptible potato clone.

Table 4: Homologs of *Solanum tuberosum* protein kinases (StPK) isolated from *R1* resistant and susceptible clones using primers EE1 and EE2 (Fig 5a)

StPK homologue	% identity to StPK-B	R1r parent 6167-2	rr parent 1024-2	R1r progeny J92-6400-A4	rr progeny J92-6400-A1	Total Clones
StPK-B	100		1			1
StPK-C	92	2		3	2	7
StPK-DÄ	82	5	3	5		13
StPK-E	91			1		1
StPK-F	91		1			1
StPK-G	86		1			1
StPK-H	90				1	1
StPK-I	89	1				1
StPK-J	91		1			1
StPK-K	94				2	2
Total		8	7	9	5	29

Ä deletion locus

A second set of primers, EE3 and EE6 (Fig 5a), of which primer EE6 is located downstream of the second exon of StPK-B, was specific enough to identify StPK-B in all analyzed plants. So, this StPK homologue is present in 87-1024-2 (identified with EE1 and EE2), in J91-6167-2 and in several tested *R1* resistant and susceptible progeny of population J92-6700, including -A16 from which the tagging population was derived. The StPK-B gene is therefore independent of the *R1* locus.

A third set of primers, EE8 and EE10 (Fig 5a), was designed on low homologous regions between StPK-A and StPK-B and specifically identified the StPK-A locus after *Bgl*II digestion of the PCR products. Analyses of all the parental genotypes used in the different crossings identified that StPK-A is present in the susceptible parent 87-1024-2 that was used to produce the starting population from which J92-6400-A16 was selected (El-Kharbotly *et al.*, 1995).

Both *Ds* transposon insertions in mutant 1000 are loci that occur solely or also in plants that do not carry the *R1* gene. Therefore, it is very unlikely that StPK-A or StPK-B are the *R1* gene itself. The *Ds* mutagenized StPK loci were designated respectively *rpr-1* and *rpr-2* (Required for *Phytophthora infestans* resistance). Both homologs cover a complete (or almost complete) serine/threonine protein kinase ORF with all conserved characteristics including a conserved intron position. The *Ds* insertions in *Rpr1* and *Rpr2* probably reduce their expression explaining the incomplete *R1* type HR resistance reaction in mutant 1000. Examples of such mutants that can produce a phenotype are given by mutation in one or two genes of a multigene family (Gilliland *et al.*, 1998). The mutations may also be semidominant due to a specific structure as described due to transposon or T-DNA insertions or inversions (Bender and Fink, 1995) (English and Jones, 1998) (Stam *et al.*, 1998).

If the StPK homologs are similar to the *Xa21* gene structure with an LRR additional to the kinase domain, then in StPK-A the *Ds* insertion in the serine/threonine kinase, 46 bp upstream of the intron, would probably form a truncated LRR protein without a functional kinase domain. This putative truncated LRR domain could possibly compete with the functional LRR-kinase genes, reducing or delaying the signal transduction to exhibit partial *P. infestans* resistance.

StPK-B contains a *Ds* insertion downstream of a serine/threonine protein kinase. For this insertion *Ds* 5' promoter activity (Rudenko *et al.*, 1994) could result in the production of an antisense RNA. Post transciptional gene silencing due to the formed aberrant RNA could result in a reduction of kinase activity making the signaling pathway leading to the *R1* type HR response less effective. This might explain the semi-dominant mutation leading to a mutated *R1* resistance phenotype in regenerant 1000. A delay in HR response could allow escape of the *P. infestans* from necrotic regions resulting in sporulation and further colonization of the infected leaves.

Transformation of StPK gene constructs conferring resistance

The StPK-B gene fragment was isolated and incorporated in binary vectors for transforming plants. A suitable vector construct with appropriate regulatory sequences including promoter sequences, terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other appropriate sequences. The StPK-B fragment encodes the kinase domain but lacks the N-terminal part including the translation initiation part. Suitable constructs (I) and (II) are shown in Fig 8. In one construct (I) a complete kinase encoding

gene was made with the StPK-B gene consisting of the DNA fragment shown as Seq ID 1, with a translation start coding for the methionine codon in frame with the open reading frame shown in Seq ID 2. In this construct-I the engineered StPK-B gene was cloned in between the constitutive CaMV 35S promoter and a nopaline synthase (Nos) terminator in the vector pBINPLUS (van Engelen et al., 1995). In another construct type (II) a translational fusion made between the STPK-B fragment of SEQ ID1and a N-terminal part of the complete gene from STPK-B or homologues genes is made. These gene fusions are cloned in between the appropriate regulatory promoter and terminator sequences in pBINPLUS

The above mentioned recombinant binary vectors are possible to construct by persons skilled in the art, including the transfer into appropriate Agrobacterium strains and checking for their stable presence in the Agrobacterium. The recombinant Agrobacterium construct containing the StPK-B overexpression cassette are transformed into potato plants by established procedures (El-Kharbotly et al., 1995). A set of transformants are regenerated and multiplied in vitro by cutting. About 5 regenerated plantlets from each individual transformant are transferred to the greenhouse.

At about 6 weeks after transferring to the greenhouse the replicated sample of the transformants are tested by the detached leaf test for *Phytophthora infestans* resistance as described in detail above. From each plant two leaflets are taken, amounting to 10 leaf samples per individual transformant. The resistance score over the replicate samples provides a quantitative estimate of the resistance reaction and allows the selection of plants significantly resistant to infection by Phytophthora.

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CLAIMS--

1. An isolated DNA sequence which encodes a protein having the amino acid sequence given in SEQ ID NO: 2 or a functionally homologous protein having an amino acid sequence showing an indentity of at least 80% to SEQ ID NO: 2, which protein confers *Phytophthora infestans* resistance on plants.

2. The DNA sequence according to claim 1, characterized in that the functionally homologous protein has an amino acid sequence showing an identity of at least 85% to SEQ ID NO: 2.

3. The DNA sequence according to claim 1 or 2, characterized in that it comprises the nucleotide sequence selected from the group consisting of:
   a) the DNA sequence given in SEQ ID NO: 1 and its complementary strand, and
   b) DNA sequences hybridizing to the sequences in (a) under stringent hybridization conditions.

4. The DNA sequence according to claim 3, characterized in that it comprises the nucleotide sequence of nucleotides 20 to 1053 of SEQ ID NO: 1.

5. The DNA sequence according to claim 3, characterized in that it comprises the nucleotide sequence of nucleotides 20 to 583 and 727 to 1053 of SEQ ID NO: 1.

6. A protein encoded by the DNA sequence of any of claims 1 to 5.

7. A recombinant vector comprising a DNA sequence under control of an appropriate promoter and regulatory elements for expression in a host cell, wherein the DNA sequence is as defined in any of claims 1 to 5.

8. Use of a DNA sequence of any of claims 1 to 5 or a recombinant vector of claim 7 for the production of a transgenic plant.

9. A host cell comprising a DNA sequence of any of claims 1 to 5 or a recombinant vector of claim 7.

10. A host cell according to claim 9 which is a plant cell.

11. A plant or any part thereof comprising a plant cell according to claim 10.

12. Seed, selfed or hybrid progeny or descendant of a plant according to claim 11, or any part thereof.

13. A method of conferring *Phytophthora infestans* resistance on a plant, comprising the steps of

i) introducing a DNA sequence of any of claims 1 to 5 or a recombinant vector of claim 7 into a cell of the plant or an ancestor thereof,

ii) regenerating plants from the obtained transgenic cells, and

iii) selecting plants exhibiting *Phytophthora infestans* resistance.

Figure. 1

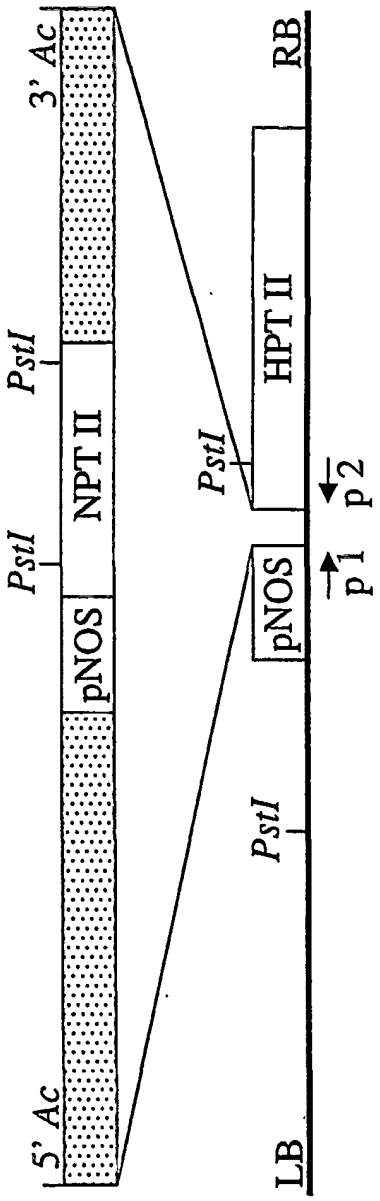
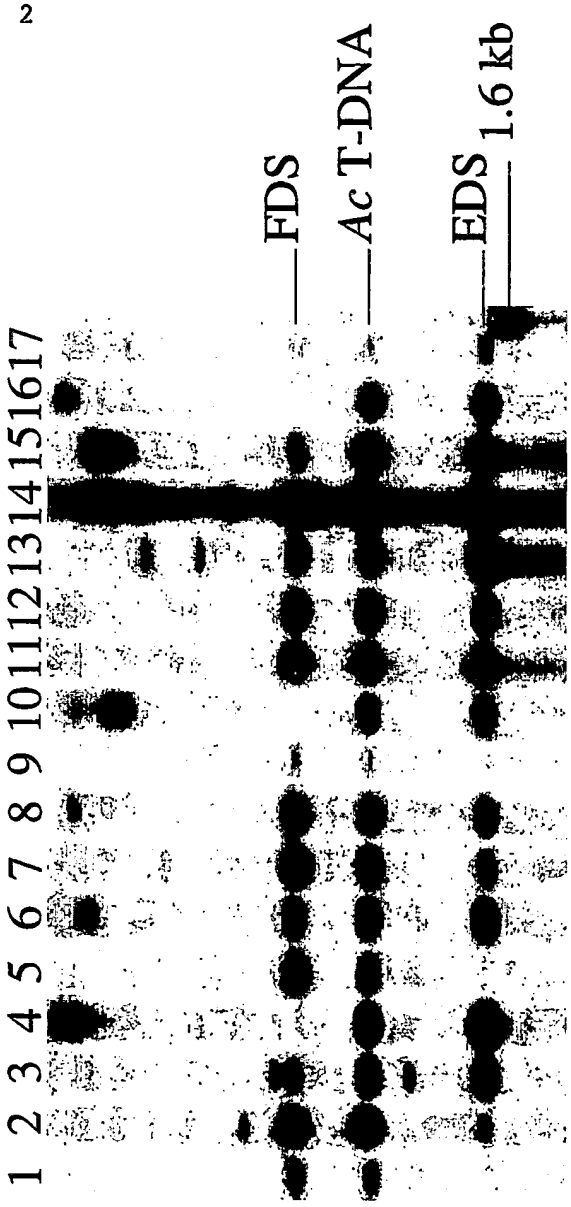


Fig. 2



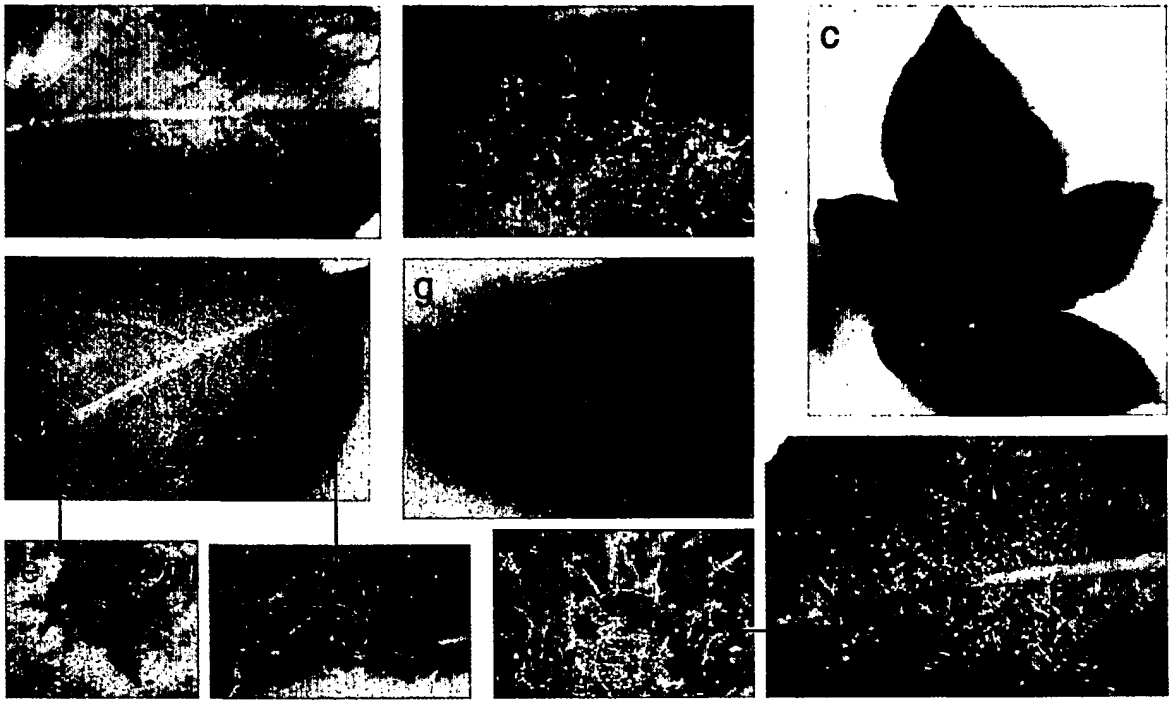


Fig. 3

Fig. 4

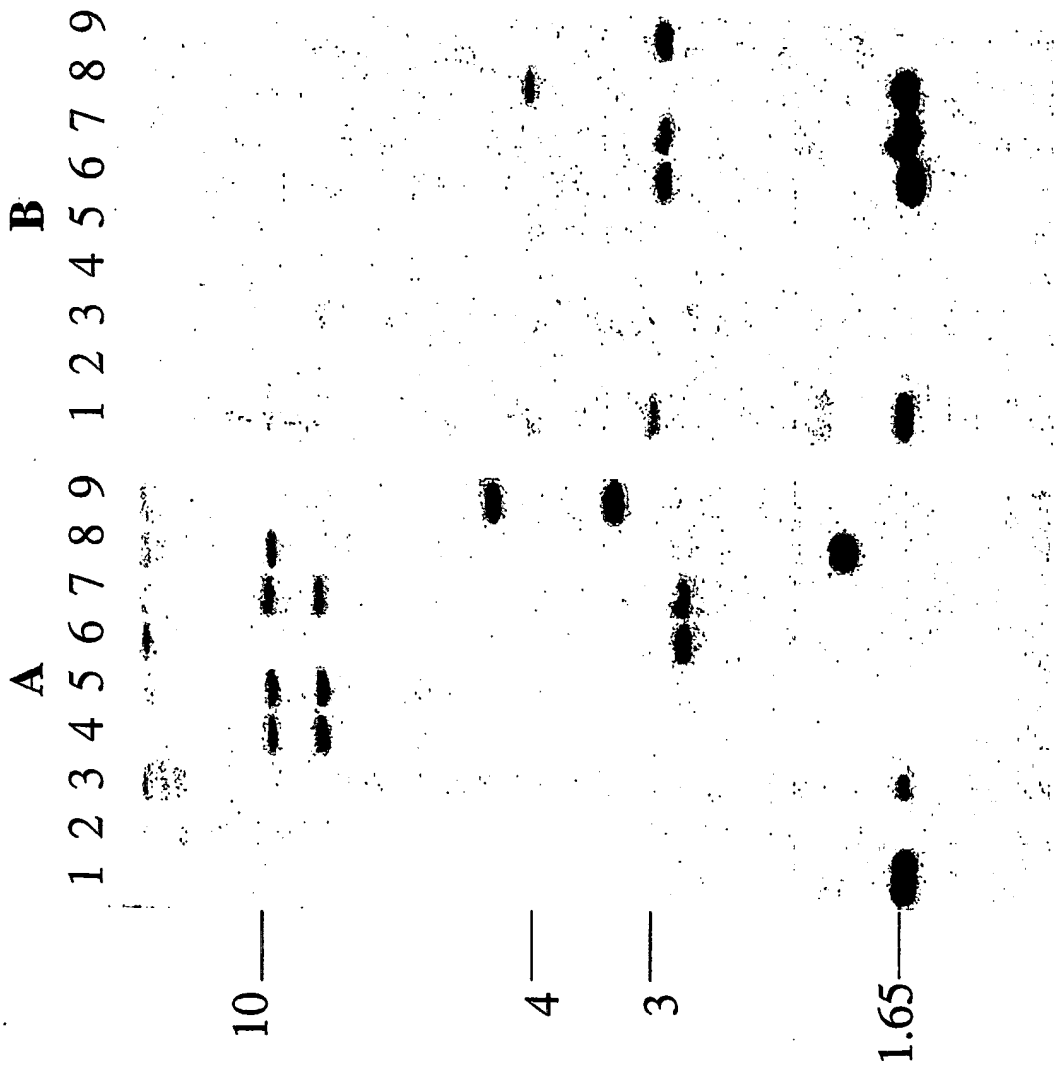
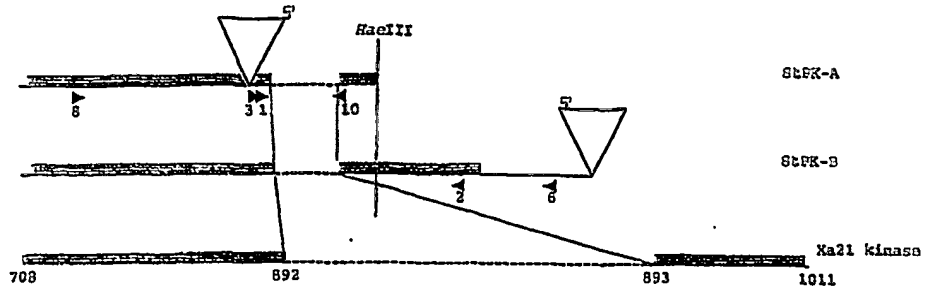


Figure 5

A



B

```
Pto             ----------------------------MGSKYSKATNSINDALSSSYLVPFESYRVPLVD 33
Ptil            MSCFSCCDDDDMHRATDNGPFMAHNSAGNNGGQRATESAQRETQTVNIQPIAVPSIAVDE 60
StPK-B          ----------------------------------------------IKTHQLVSYHE 11
StPK-A          ----------------------------------------------IKTNQLISYHE 11
Xa21 kinase     ------------------------------------------------------------

                                * *I   *          II*                *III
Pto             LEEATNNFDH-KFLIGHGVFGKVYKGVLRDGAKVALKRRTPESSQGIEEFETEIETLSFC 92
Ptil            LKDITDNFGS-KALIGEGSYGRVYHGVLKSGRAAAIKKLD-SSKQPDREFLAQVSMVSRL 118
StPK-B          IQQATNNFDDKSNLIGEGSSGSVYKGILSIGTVVAIKVLDLENEQVCKRFDTECKVMRNV 71
StPK-A          IQQVTNNFDG-SNLIGEGSSGSVYKGTLSSGTTVAIKVLDLENEQVCKRFXTECEVMRNV 70
Xa21 kinase     --------FAPTNLLGSGSFGSVYKGKLNIQDHVAVKVLKLENPKALKSFTAECEALRNM 52

                    IV                          V
Pto             RHPHLVSLIGFCDERNEM-----ILIYKYMENGNLKRHLYG-----SDLPTMSMSWEQRL 142
Ptil            KDENVVELLGYCVDGGFR-----VLAYEYAPNGSLHDILHGRKGVKGAQPGPVLSWAQRV 173
StPK-B          RHRNLVPVITTCSSDYIR-----GFVMPIMPNGSLENWLYKED--------RHLNLHQRV 118
StPK-A          RHRNLVPVITTCSSDYXX-----AFVLKYMSXGSHENWLYREV--------RHLNLLQRV 117
Xa21 kinase     RHRNLVKIVTICSSIDNRGNDFKAIVYDFMPNGSLEDWIHPETN--DQADQRHLNLHRRV 110

                                  VI*     *            ***VII
Pto             EICIGAARGLHYLHTRA---IIHRDVKSINILLDENFVPKITDFGISKKGTELDQTHLST 199
Ptil            KIAVGAAKGLEYLHEKAQPHIIHRDIKSSNILLFDDDVAKIADFDLSNQAPDMAARLHST 233
StPK-B          TVMLDAAMAVEYLHHCHVAPIVHCDLKPANVLLDEDMVAHVGDFGISKILAISKSMAYTE 178
StPK-A          TVMLDAAMAIEYLHHGNDTVIVHCDINPANVLLDEDMVAHVGDFGISKILAASKSLTQTE 177
Xa21 kinase     TILLDVACALDYLHRHGPEPVVHCDIKSSNVLLDSDMVAHVGDFGLARILVDGTSLIQQS 170

                     VIII   *           *  IX*
Pto             V----VKGTLGYIDPEYFIKGRLTEKSDVYSFGVVLFEVLCARSAIVQSLPREMVNLAEW 255
Ptil            R----VLGTFGYHAPEYAMTGQLSSKSDVYSFGVVLLELLTGRKPVDHTLPRGQQSLVTW 289
StPK-B          T-----LGTLGYIAPEYGSEGIVSASGDVYSYGIMLMEVLTKRRPTDEDICNENLDLRKW 233
StPK-A          T-----LGTLGYIAPEYGSEGIVSASGDVYSYGIMLMEVLTKRR---------------- 216
Xa21 kinase     TSSMGFIGTIGYAAPEYGVGLIASTHGDIYSYGILVLEIVTGKRPTDSTFR-PDLGLRQY 229

                X                                                   XI   *
Pto             AVESHNNGQLEQIVDPNLADKIR--------------PESLRKFGDTAVKCLALSSEDRP 301
Ptil            ATPRLSEDKVKQCVDARLNTDYP--------------PKAIAKMAAVAALCVQYEADFRP 335
StPK-B          ITQSFS-GSMMDVVDANLFSEEEQITCKS--------EMCIASMIELALDCTKKMPESRV 284
StPK-A          ------------------------------------------------------------
Xa21 kinase     VELGLH-GRVTDVVDTKLILDSENWLNSTNNSPCRRITECIVWLLRLGLSCSQELPSSRT 288


Pto             SMGDVLWKLEYALRLQESVI 321
Ptil            NMSIVVKALQPLLPRPVPS- 354
StPK-B          TMKEVVKRLNKIK------- 297
StPK-A          --------------------
Xa21 kinase     PTGDIIDEL----------- 297
```

FIG 6(A)
SEQ ID NO:1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AGTGGCCATA AAGGTTCTGG ATTTGGAAAA TGAGCAAGTA TGCAAGAGGT  200
TTGATACCGA ATGCAAAGTG ATGAGAAATG TTAGACACAG AAATCTTGTT  250
CCAGTGATCA CTACATGTTC TAGTGACTAT ATAAGAGGCT TTGTTATGCC  300
AATTATGCCC AATGGAAGTC TTGAGAATTG GCTGTACAAA GAAGATCGCC  350
ACTTGAACCT TCATCAAAGA GTAACTGTAA TGCTTGATGC AGCTATGGCA  400
GTTGAATATC TACATCATTG TCATGTTGCT CCAATAGTTC ATTGCGACCT  450
AAAGCCAGCC AACGTTCTTT TGGATGAAGA TATGGTGGCT CATGTTGGTG  500
ATTTTGGAAT CTCTAAAATT TTAGCTATAA GCAAGTCCAT GGCCTATACC  550
GAGACATTGG GTACTCTTGG ATACATTGCA CCAGGTATAA AAAATCTACC  600
CTCTTTGATT TTCTCTTATC ATAATTAAAC CTCTCTAAAT TCTACCAGTA  650
AGAAAAAGCA AGGATTTATT TATGCAGAAT TATTGTTGTA TTTCAATTGA  700
GTAACTTTTC TTCAATTCTT TTCTAAGAAT ATGGCTCGGA GGGAATAGTG  750
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GACCAAAAGA CGGCCAACAG ATGAAGATAT ATGCAATGAA AATCTTGACC  850
TGAGGAAATG GATAACACAA TCATTTTCAG GGAGTATGAT GGATGTTGTG  900
GATGCTAATC TTTTTTCTGA GGAAGAACAA ATTACTTGTA AAAGTGAAAT  950
GTGCATAGCC TCCATGATAG AATTGGCTTT AGACTGCACA AAGAAAATGC 1000
CAGAATCAAG AGTAACCATG AAAGAAGTAG TCAAGAGGCT TAACAAAATC 1050
AAGAACACAT TTTTGGAAAT GTAGAAGTGA TCAGCATCTC TTTCTGATCT 1100
GCAAGTTAAC TTGTTGCTTT TTGTTTACTG GTTTCTTTAG TAAAGGCGTA 1150
TGTACTACTC GAAGTCATGT ATTGTTTATA CTTTAGAGTG TTGCATTTTG 1200
GAGAAGAAAG GCATTGTTCC GAGGAAGTGG TAATATATCA TCTCTTTATA 1250
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ACTTGTTCG  1309
```

Fig. 6(B)

SEQ ID NO:2
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DLENEQVCKRFDTECKVMRNVRHRNLVPVITTCSSDYIRGFVMPIMPNGS

LENWLYKEDRHLNLHQRVTVMLDAAMAVEYLHHCHVAPIVHCDLKPANVL

LDEDMVAHVGDFGISKILAISKSMAYTETLGTLGYIAPEYGSEGIVSASG

DVYSYGIMLMEVLTKRRPTDEDICNENLDLRKWITQSFSGSMMDVVDANL

FSEEEQITCKSEMCIASMIELALDCTKKMPESRVTMKEVVKRLNKIK

Fig.7

Seq ID NO:3

StPK - A

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CATAAAGGTT CTGGATTTGG AAAATGAGCA AGTATGCAAG AGGTTTGRTA  200
CAGAATGCGA AGTGATGAGA AATGTCAGAC ATAGAAATCT TGTTCCAGTG  250
ATTACTACTT GTTCTAGTGA CTATAYARCA GCCTTTGTYC TGAAATATAT  300
GTCAWATGGG AGTCACGAAA ATTGGTTGTA CAGAGAAGTT CGCCACTTGA  350
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TATCTACATC ATGGCAATGA CACTGTGATA GTTCATTGCA GACATAAACC  450
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TGAGTTAGTT CTTTTTCATT TTCGTTCTTT TAAGAGTACG GCTCAGAAGG  750
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Seq ID NO:4

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TGATAGAATT GGGTTTAGAC TGCACAAAGA AAATGCCAGA ATCAAGA    447
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Seq ID NO:5

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GACTAAATAA AAAGCAAGTC TTTGTTATTA GTACAAGCAT TATTGTTATA  150
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GAAATCTGCA TAGCCTCCAT GATAGAATTG GCTTTAGATT GCACAAAGAA  350
AATGCCAGAA TCAAGA 366
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Seq ID NO:6

StPK - E

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Fig.7 (cont.) 9/11

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TGAATAACTG TTTTTTTCTC AACCCTTTTC TATGAATATG GCTCGGAGGG   200
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AGGTTTTGAC CAAGAGAAGG CCAACAGATG AAGAGATATG CAATGAAACT   300
CTTGACTTGA GGAAATGGAT CACACAATCA TTTTCAGGGA GTATGATGGA   350
CGTTGTGGAT GCCAATCTTT TCTCCGAGGA AGAACAGATC ACTTCAGAAA   400
GTGAAATCTG CATTGCGTCC ATGATAGAAT TGGGTCTAGA CTGCACAAAG   450
AAAATGCCAG AATCAAGA     468
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Seq ID NO:7

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ATGCCAATCT TTTTTACGAG GAAGAACAAA CCACTAGTAA AAGTGAAATC   300
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AGAATCAAGA  360
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Seq ID NO:8

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Seq ID NO:9

StPK - H

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Seq ID NO:10

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Fig.7 (cont.)

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CAATCTTTTT TGTGAGGAAG TACAAATCAC TTGTAAAAGT GAAATGTGCC 400
TAGCCTCCAT GATAGAATTG GCTTTAGATT GCACAAAGAA AATGCCAGAA 450
TCAAGA 456

Seq ID NO:11

StPK - J

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AAAGCAACCT TTTATTTATG CAGAATTATT GTTGTATTTC AATTGAATTA 150
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CGAAAAGAAG GCCAACAGAT GAAGAGATAT GCAATGAAAA TCTTGACCTG 300
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AATCAAGA 458

Seq ID NO:12

StPK - K

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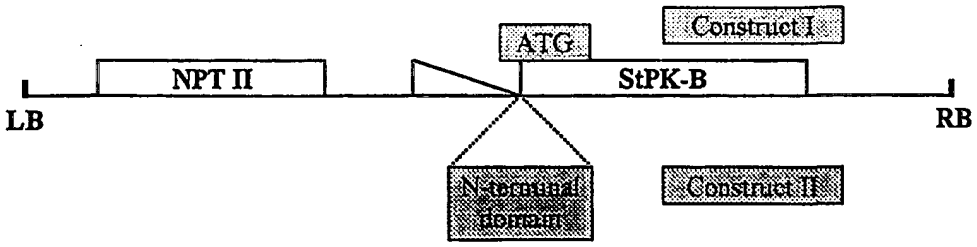
CTTGACTTGA GGAAATGGAT CACACAATCA TTTTCAGGGA GTATGATGGA 350
TGTTGTGGAT GCCAATCTAT TTTCTGCGGA AGAACAAATC ACTAGTAAAA 400
GTGAAATGTG CATAGCCTCC ATGATAGAAT TGGCTTTAGA CTGCACAAAG 450
AAAATGCCAG AATCAAGA 468

Figure 8:


ATG
Construct I
NPT II
StPK-B
LB
RB
N-terminal domain
Construct II

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Declaration under Rule 4.17:
as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii)) for the following designations AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG)

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(54) Title: DNA SEQUENCES ENCODING PROTEINS CONFERRING PHYTOPHTHORA INFESTANS RESISTANCE ON PLANTS

(57) Abstract: Genomic sequences encoding *Phytophthora infestans* resistance proteins are provided herein. Specifically, sequences from potato required for *P. infestans* resistance have been cloned and sequence provided, together with the encoded amino acid sequence. DNA encoding the amino acid sequence or amino acid sequences showing a significant degree of homology thereto may be introduced into plant cells and the encoded polypeptide expressed, conferring *P. infestans* resistance on plants comprising such cells and descendants thereof.

INTERNATIONAL SEARCH REPORT

Int'l Application No
PCT/NL 01/00804

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N9/12 C12N15/82 C12N15/63

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, SEQUENCE SEARCH, EMBL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	VAN ENCKEVORT L.J.G.: "Identification of potato genes involved in Phytophthora infestans resistance by transposon mutagenesis (thesis)" 21 November 2000 (2000-11-21) , WAGENINGEN UNIVERSITY, NL XP002210496 ISBN: 90-5808-343-8 page 85, paragraph 3 -page 93, paragraph 4 ---	1-13
A	VAN ENCKEVORT L.J.G. ET AL.: "Selection of independent Ds transposon insertions in somatic tissue of potato by protoplast regeneration" THEORETICAL AND APPLIED GENETICS, vol. 101, September 2000 (2000-09), pages 503-510, XP000926420 the whole document --- -/—	1-13

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

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"P" document published prior to the international filing date but later than the priority date claimed
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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 August 2002	10/09/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Schönwasser, D

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	EL-KHARBOTLY A. ET AL.: "Localization of Ds-transposon containing T-DNA inserts in the diploid transgenic potato: linkage to the R1 resistance gene against Phytophthora infestans (Mont.) de Bary" GENOME, vol. 39, no. 2, April 1996 (1996-04), pages 249-257, XP000926428 cited in the application the whole document	1-13
A	TARCHINI R. ET AL.: "The Complete Sequence of 340 kb of DNA around the Rice Adh1-Adh2 Region Reveals Interrupted Colinearity with Maize Chromosome 4" THE PLANT CELL, vol. 12, no. 3, March 2000 (2000-03), pages 381-391, XP002168485 cited in the application page 382, column 2, line 10 -page 383, column 2, line 6; table 1 page 389, column 2, paragraph 2	1-6
A	KANEKO T. ET AL.: "Structural analysis of Arabidopsis thaliana chromosome 5.V. Sequence features of the regions of 1,381,565 bp covered by twenty one physically assigned P1 and TAC clones" DNA RESEARCH, vol. 5, no. 1, 30 April 1998 (1998-04-30), pages 131-145, XP000926429 cited in the application page 135: figure legend, second line from the bottom; page 139: line 8 of the first table (MPL12)	1-6
A	DATABASE EMBL 'Online! 28 July 1999 (1999-07-28) ALCALA J. ET AL.: "Generation of ESTs from tomato carpel tissue; EST267901 tomato ovary, TAMU Lycopersicon esculentum cDNA clone cLED34E19, mRNA sequence" Database accession no. AI898458 XP002168486 abstract	1-6

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/NL 01/00804

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SUNDARESAN V. ET AL.: "PATTERNS OF GENE ACTION IN PLANT DEVELOPMENT REVEALED BY ENHANCER TRAP AND GENE TRAP TRANSPOSABLE ELEMENTS" GENES AND DEVELOPMENT, vol. 9, no. 14, 15 July 1995 (1995-07-15), pages 1797-1810, XP000674520 ISSN: 0890-9369 the whole document	1-13
A	MEISSNER R. ET AL.: "A new model system for tomato genetics" THE PLANT JOURNAL, vol. 12, no. 6, 1997, pages 1465-1472, XP002096585 ISSN: 0960-7412 the whole document	1-13

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